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Small RNA-Directed Silencing: The Fly Finds Its Inner Fission Yeast?

Several recent studies demonstrate that piRNAs guide Piwi protein to repress transposon transcription in fly ovaries, much as fission yeast use siRNAs to silence repeat sequences. Still mysterious though is how Piwi targets euchromatic transposons for silencing, but not the specialized heterochromatic loci that produce piRNA precursors.

Daniel Tianfang Ge and Phillip D. Zamore

Fungi, plants, and animals devote considerable resources to thwart transposable elements from increasing their numbers or moving to new genomic locations, particularly in germ cells. In fungi and plants, small interfering RNAs (siRNAs) act via the RNA interference (RNAi) pathway to silence transposons and other types of repetitive DNA. In contrast, animals use PIWI-interacting RNAs (piRNAs), a class of small silencing RNAs distinct from siRNAs, to silence germline transposons and ensure fertility. Like siRNAs and the mRNA-regulating microRNAs (miRNAs), piRNAs direct Argonaute proteins to silence complementary nucleic acid targets. Unlike siRNAs and miRNAs, piRNAs guide a specialized sub-class of Argonautes, the PIWI proteins, which are found exclusively in animals and nearly always in the germline or germline-related cells.

In *Drosophila*, piRNAs bind three different PIWI proteins: *P*-element-*i*nduced *w*impy *t*estes (Piwi), Aubergine (Aub), and Argonaute3 (Ago3). Aub and Ago3 act strictly in the ovary and testis germline, where they silence transposons by destroying their RNA transcripts. In contrast, Piwi resides in the nucleus, where it represses transposons in both germ cells and their supporting somatic cells [1–3]. Now, four papers demonstrate that Piwi silences transposons, at least in part, by repressing their transcription [4–7]. These genome-scale studies support and extend earlier evidence that Piwi directs transcriptional silencing in the nucleus [3,8,9]. By depleting Piwi in the ovarian germline [5,6], ovarian somatic follicle cells [6], or cultured, immortalized ovarian somatic cells (OSCs) [4], or by inserting ectopic piRNA target sites into the fly genome [7], all four studies find that piRNAs guide Piwi to its target loci, where it recruits enzymes that establish repressive heterochromatin (Figure 1A). The papers generally support the view that piRNAs tether Piwi to nascent transcripts: RNA is required for Piwi to co-immunoprecipitate with chromatin [7] and with proteins known to bind nascent RNA [5]. Piwi bound to nascent RNA via its piRNA guide appears to recruit Su(var)3-9 [7], a histone methyltransferase that methylates histone H3 on lysine 9. These 'H3K9me3' marks bind heterochromatin protein 1 (HP1, officially named Su(var)205), generating chromatin that is refractory to transcription, as reflected by reduced occupancy with RNA polymerase II (pol II) [7]. Supporting this view, depletion of Piwi reduces the amount of H3K9me3 [4–6,9] and HP1 [9] and increases the amount of RNA pol II [4,5] and nascent transcripts [4,6,8] at transposon sequences.

These findings call to mind the mechanism by which the RNAi pathway silences repetitive sequences in the fission yeast, *Schizosaccharomyces pombe*. siRNAs bound to *S. pombe* Ago1 guide the 'RITS' complex to nascent transcripts from transposon-like repeats near the centromere, where it recruits proteins that establish repressive

heterochromatin [10] (Figure 1B). Unlike fission yeast, whose siRNAs derive from the silenced loci themselves, transposon silencing in flies requires transcription of both a trigger locus — a 'piRNA cluster' — and a transposon target locus. The model that has emerged since 2006 maintains that piRNAs originate from heterochromatic, transposon-rich piRNA clusters, which record the fly's history of transposon invasion. The new findings suggest that Piwi-bound piRNAs bind the nuclear transcripts of euchromatic transposons, initiating their silencing. Does Piwi also ensure the heterochromatic character of piRNA clusters? How are the clusters transcribed rather than silenced, and why do their transcripts generate piRNAs when RNA from other genes does not?

In Piwi-depleted OSCs, the amount of H3K9me3 is unaltered for heterochromatic loci, where most piRNA clusters reside [4]. Similarly, germline loss of Armitage — a protein required for wild-type Piwi abundance and localization — has no effect on the transcription of at least two heterochromatic elements [11]. Yet Piwi depletion in the ovarian germline shows a general decrease of H3K9me3 in heterochromatic loci [5], although a similar experiment by Hannon and colleagues failed to detect such a change in the chromatin of germline-active piRNA clusters [6]. At present, it is not known if these discrepant results reflect the different tissues or cell populations studied, the difficulty in accurately assigning highly repetitive heterochromatic sequences to specific genomic locations, or a difference in the chromatin status of piRNA clusters and the surrounding heterochromatin.

Piwi helps maintain H3K9me3 marks on euchromatic transposon copies but may play a different role for the transcriptionally active, heterochromatic, piRNA-producing clusters. For these loci, Piwi may recruit proteins that facilitate the processing of piRNA precursor transcripts, such as Rhino [12], Cutoff [13] or UAP56 [14] (Figure 1A). Without Piwi, clusters may still be transcribed, but the resulting RNA may fail to be exported to the perinuclear nuage for conversion into piRNAs. Such a model helps explain why Piwi depletion leads to nuclear accumulation of transcripts containing transposon sequences

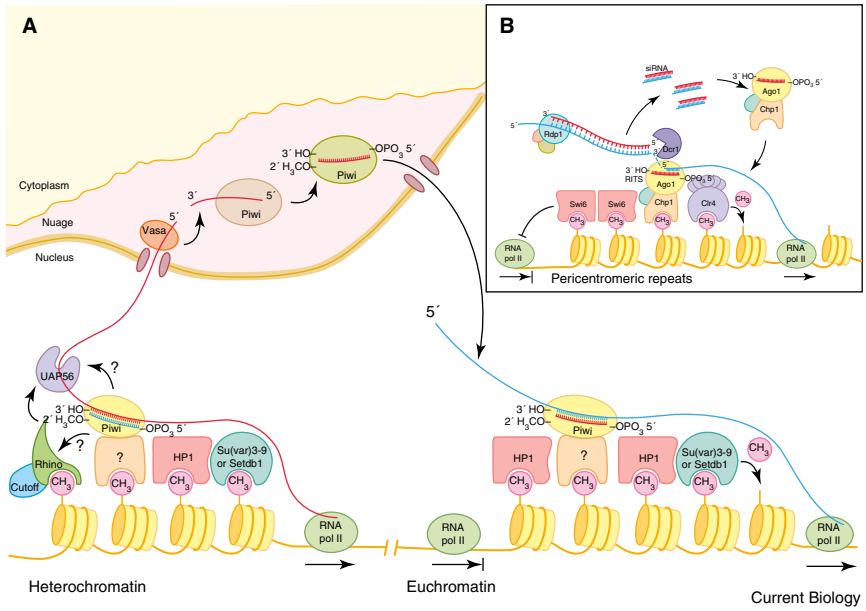

Figure 1. Small RNA-guided transcriptional silencing.

(A) Current evidence suggests that only Piwi loaded with a mature piRNA is allowed to enter the nucleus, where it binds to nascent transposon transcripts in euchromatin, recruiting Su(var)3-9 or Setdb1 to methylate nearby histone H3 at lysine 9. HP1 binds to H3K9me3 and generates chromatin that is refractory to transcription. In *Drosophila* heterochromatin, Piwi bound to nascent piRNA cluster transcripts may recruit Rhino, Cuff or UAP56 to facilitate export of the transcripts to cytoplasmic sites of piRNA production. (B) In *S. pombe*, siRNA-guided Ago1 binds nascent transcripts and Chp1 associates with chromatin at pericentromeric repeats. Ago1 and Chp1 are components of the RITS complex. RITS recruits Clr4 to methylate nearby histone H3 at lysine 9. Swi6 binds to H3K9me3 and generates transcriptionally repressed chromatin. The nascent transcript may be cleaved by Ago1 and converted into double-stranded RNA by Rdp1. Dcr1 generates siRNA duplexes and loads them into Ago1 to target additional transcripts.

[3], why steady-state level of transposon-containing transcripts increases more than nascent transcription output [6], and why mature piRNA abundance, including piRNAs presumably bound to Ago3, declines when transposon-containing transcripts accumulate [6].

In *S. pombe*, silencing can spread beyond the region of heterochromatin initially established by Ago1-bound siRNAs. In Piwi-depleted OSCs, protein-coding genes adjacent to transposons show decreased H3K9me3 and increased transcription [4], suggesting that these regions are normally silenced by the spreading of heterochromatin beyond transposon boundaries. Such spreading might result from HP1 recruiting the histone methyltransferase Su(var)3-9 or Setdb1, which both deposit H3K9me3 marks on nearby nucleosomes, in turn recruiting more HP1 [15]. This process would not require piRNAs complementary to the sequences flanking the transposons. While the model is appealing, data from Toth and colleagues suggest that expression of genes adjacent to piRNA-silenced loci is unaffected by loss of Piwi [5], and argue that much of the observed increase in expression of protein-coding genes reflects the induction of stress responses triggered by transposon activation and associated DNA damage.

To date, all RNA silencing pathways — that is, those mediated by Argonaute proteins and directed by small RNA guides — derive their nucleic acid binding specificity from a specialized guide region, the seed. Argonaute proteins create the seed by pre-organizing guide nucleotides 2–8 in a geometry resembling one strand of an RNA helix. Given the remarkable structural conservation of Argonaute proteins from bacteria, fungi, and animals, the field has assumed that all Argonaute proteins derive their binding specificity from complementarity between the seed and target sequences. Supporting the view that

the general biochemical properties of Argonaute proteins are conserved among PIWI proteins, fly and mouse PIWIs cleave their target RNAs at the same phosphodiester bond as Argonaute proteins acting in the miRNA and siRNA pathways [16,17]. Moreover, mutation of the predicted 5′-phosphate-binding pocket, which anchors the small RNA guide to an Argonaute protein, blocks small RNA binding by Piwi *in vivo* [5]. However, Lin and colleagues find that target mutations that disrupt pairing with either the piRNA seed or regions outside the seed have similar effects *in vivo* [7]. Given that the endonuclease activity of Piwi is dispensable for Piwi to silence transposons [2,4,18], their data suggest that Piwi uses an alternative, non-seed mechanism to bind its targets. Clearly, rigorous quantitative biochemical analysis will be required to test this proposal.

The paradoxical requirement for transcription to silence transcription of a locus means that silencing is a quantitative process: transcription of repetitive sequences can be reduced but not eliminated if the loci are to remain silent. Thus, additional mechanisms such as post-transcriptional RNA destruction are likely required to achieve complete silencing. Indeed, although many piRNAs appear to be bound by both Piwi and Aub in the *Drosophila* germline, transcriptional silencing by Piwi-bound piRNAs is not redundant with post-transcriptional RNA destruction by piRNAs bound to Aub. In fact, only some transposon families recruit more RNA pol II [5] or increase transcription [6] when Piwi is depleted in the ovary using RNAi.

The contradictory result that in *piwi* mutant flies all transposon families but one recruit more RNA pol II [7] needs to be interpreted with caution. Traditionally, more weight has been placed on observations made using *bona fide* genetic mutations than RNAi. However, *piwi* mutants have rudimentary ovaries, making comparisons even to the small ovaries of newly eclosed females challenging. Although adult flies have little if any Piwi protein outside the gonads, the use of whole flies instead of isolated ovaries complicates the analysis of chromatin immunoprecipitation (ChIP) data for RNA pol II, HP1, and histone methylation marks [7], because deconvoluting the germline signal from the broader somatic signal is not possible.

Mapping the genomic locations of Piwi protein is quite challenging [4,19], particularly because Piwi appears to be tethered to the chromatin via nascent RNA transcripts. To accomplish this, Lin and colleagues employed an atypical ChIP protocol in which isolated nuclei were cross-linked with formaldehyde, rather than the more standard method of cross-linking the cells or tissue prior to disrupting the cell membrane. Moreover, their protocol omits ionic detergents during nuclear lysis, raising the theoretical possibility that some Piwi binding events may have occurred in the lysate rather than *in vivo*.

Why do flies need two independent yet seemingly overlapping mechanisms to protect their germline? Perhaps the requirement for nascent RNA to recruit Piwi to euchromatic transposon insertions limits the effectiveness of the pathway, with Aub subsequently mopping up the spillover from Piwi-mediated silencing. If true, this model suggests that all small RNA silencing pathways that use nascent RNA as staging for modifying nearby chromatin will require a parallel post-transcriptional mechanism to ensure complete silencing of transposons and repetitive RNAs. It is tempting to speculate that the reported post-transcriptional silencing activity of yeast Ago1 may provide such a backup function in *S. pombe* [20].

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